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Plant defense genes are regulated by ethylene

(stress responses/plant hormone/wounding/RNA blot analysis)

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ABSTRACT **One of the earliest detectable events during plant–pathogen interaction is a rapid increase in ethylene biosynthesis. This gaseous plant stress hormone may be a signal for plants to activate defense mechanisms against invading pathogens such as bacteria, fungi, and viruses. The effect of ethylene on four plant genes involved in three separate plant defense response pathways was examined; these included (*i* and *ii*) genes that encode L-phenylalanine ammonia-lyase (EC 4.3.1.5) and 4-coumarate:CoA ligase [4-coumarate:CoA ligase (AMP-forming), EC 6.2.1.12], enzymes of the phenylpropanoid pathway, (*iii*) the gene encoding chalcone synthase, an enzyme of the flavonoid glycoside pathway, and (*iv*) the genes encoding hydroxyproline-rich glycoprotein, a major protein component(s) of plant cell walls. Blot hybridization analysis of mRNA from ethylene-treated carrot roots reveals marked increases in the levels of phenylalanine ammonia-lyase mRNA, 4-coumarate CoA ligase mRNA, chalcone synthase mRNA, and certain hydroxyproline-rich glycoprotein transcripts. The effect of ethylene on hydroxyproline-rich glycoprotein mRNA accumulation was different from that of wounding. Ethylene induces two hydroxyproline-rich glycoprotein mRNAs (1.8 and 4.0 kilobases), whereas wounding of carrot root leads to accumulation of an additional hydroxyproline-rich mRNA (1.5 kilobases). These results indicate that at least two distinct signals, ethylene and a wound signal, can affect the expression of plant defense-response genes.**

Resistance of plants to disease involves inducible defense mechanisms (1). The enzymes L-phenylalanine ammonia-lyase (EC 4.3.1.5) (PAL), 4-coumarate CoA ligase (4-coumarate:CoA ligase AMP-forming, EC 6.2.1.12; 4-CL), and chalcone synthase (EC 2.3.1.74) (CHS) play a central role in the inducible defense response (2, 3). PAL is the first enzyme in the general pathway of phenylpropanoid metabolism in plants and catalyzes the elimination of ammonia from phenylalanine to produce cinnamic acid. 4-CL is the last enzyme in the general pathway and catalyzes the activation of cinnamic acids to the corresponding CoA thioesters. These compounds can serve as substrates for several important biochemical pathways in plants including the formation of phenolic compounds, such as lignin, a structural component of plant cell walls, and plant antibiotics called phytoalexins (2–4). CHS catalyzes the formation of naringenin chalcone, which is an intermediate in the synthesis of flavanoids and phytoalexins (4). Both lignin and phytoalexins are induced during pathogen attack and play important roles in the plant defense response (2–6).

Induction of PAL, 4-CL, and CHS enzyme activity is correlated with increased resistance to pathogenic infection (7–9). Inhibition of PAL enzyme activity with a specific PAL inhibitor, aminooxy-β-phenylpropionic acid, results in greatly reduced levels of phenolic compounds and is correlated with an increase in susceptibility of plants to infection (9). Coordinate induction of PAL, 4-CL, and CHS mRNAs occurs in response to pathogen infection (7, 8). Similarly, treatment of plants or plant cell cultures with active fungal cultures or elicitors (fungal or plant cell wall extracts) induces the accumulation of PAL, 4-CL, and CHS mRNAs (7, 8, 10–14). The induction of these mRNAs has been shown to occur via an increase in the rate of specific transcription of these genes (15, 16).

Several other defense response proteins including hydroxyproline-rich glycoproteins (HRGPs) (17–19), chitinase (20–23), and pathogenesis-related proteins (24, 25) are induced by pathogen attack or by elicitor treatment. HRGPs are the major structural proteins of plant cell walls (26–28). The accumulation of HRGPs occurs in response to wounding (29, 30) or infection (17–19, 31–33) and is correlated with the expression of increased disease resistance (31–36). Consistent with these results, rapid accumulation of HRGP mRNAs occurs in response to fungal infection (33). The exact role of HRGPs in the defense response is not clear, but they may act as structural barriers, provide a matrix for the deposition of lignin, and/or as specific agglutinins of microbial pathogens (34–36).

One of the earliest events during plant–pathogen (elicitor–plant cell) interaction is a rapid increase in ethylene biosynthesis (37–41). Ethylene is a gaseous plant hormone that is involved in the regulation of numerous plant processes including growth, development, and fruit ripening (42). Ethylene is known to regulate the accumulation of specific plant mRNAs, as well as the rate of transcription of specific plant genes (23, 43–46). The molecular mechanism of ethylene action is unknown, but its biosynthesis in plants occurs through *S*-adenosylmethionine and 1-aminocyclopropane-1-carboxylic acid (ACC) intermediates (42). A variety of biological stresses, such as viral or fungal infection and treatment with elicitors, rapidly induce ethylene production in plants (37–42). This ethylene increase is the result of an increase in the activity of ACC synthase (*S*-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14), the rate-limiting ethylene biosynthetic enzyme. The induction of ACC synthase is the most rapid of any enzyme induction in response to elicitor treatment (39). To demonstrate a more direct link between ethylene induction and the expression of plant defense responses, we have examined the effect of ethylene on the regulation of PAL, 4-CL, CHS, and HRGP genes. We report that ethylene has a dramatic effect on the accumulation of mRNAs for these plant defense-response genes.

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Abbreviations: PAL, phenylalanine ammonia-lyase; 4-CL, 4-coumarate:CoA ligase (AMP forming); CHS, chalcone synthase; HRGP, hydroxyproline-rich glycoprotein; ACC, 1-aminocyclopropane-1-carboxylic acid.

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MATERIALS AND METHODS

Treatment of Plant Materials. Whole carrot roots obtained from the local market were placed in 4-liter jars and allowed to equilibrate at 20°C in a stream of moist air. After 24 hr, the roots were continuously flushed at a rate of 100 ml/min with either 10 ppm ethylene in hydrocarbon-free air, 10 ppm ethylene in oxygen or in hydrocarbon-free air, or oxygen alone. At various times during the treatment period, groups of at least four to six carrots per treatment condition were removed, frozen in liquid nitrogen, and placed at −70°C. Alternately, a cork borer was used to remove a plug of tissue from each of at least six individual roots per treatment condition. For wounding experiments the upper 1–2 mm of the root tissue (peel) was quickly removed and discarded, and then an additional 1–2 mm of tissue was peeled directly into liquid nitrogen (t_0). The peeled roots were placed in jars, and a stream of moist hydrocarbon-free air was continuously passed over them at 100 ml/min. At various times after peeling, groups of four or five wounded roots were removed, the upper 1–2 mm of tissue was peeled into liquid nitrogen, and the remaining root tissue was discarded.

Carrot protoplasts were prepared from the carrot cell line WOO1C (47). Briefly, carrot cells grown in Murashige and Skoog (MS) medium (48) containing 0.1 mg of 2,4-dichlorophenoxyacetic acid per liter were pelleted by centrifugation 1000 × *g* for 5 min, resuspended in 2% Driselase (Plenum, Hackensack, NJ) 10 mM NaOAc, pH 5.5/50 mM $CaCl_2$/0.6 M sorbitol and incubated for 3–4 hr at 25°C. The protoplasts were washed twice by pelleting and resuspending in MS medium containing 0.6 M sorbitol. Protoplasts were placed in plastic tissue culture flasks (T-175, Falcon products) and incubated at 25°C in the dark. At various times after the protoplast stage, carrot cells were pelleted and frozen in liquid nitrogen. Polyadenylylated RNA was prepared from carrot protoplast as described below.

Preparation of RNA. Total nucleic acids were prepared from frozen carrot tissues. Briefly, frozen root tissue was placed in extraction buffer (1:1 mixture of 150 mM NaCl/50 mM Tris, pH 7.5/5 mM EDTA/25 mM dithiothreitol/2% $NaDodSO_4$ and phenol/chloroform/isoamyl alcohol, 24:24:1) and macerated with a polytron mixer (Brinkman). The mixture was centrifuged; the aqueous phase was then removed and reextracted with an equal volume of phenol/chloroform/isoamyl alcohol. Nucleic acids were precipitated by raising the NaCl concentration to 0.4 M and adding 2.5 vol of cold ethanol. After several hours at −20°C, nucleic acids were pelleted, resuspended in TE buffer (10 mM Tris, pH 7.5/1 mM EDTA) and reprecipitated. Polyadenylylated RNA was prepared by oligo(dT)-cellulose chromatography as described by Theologis *et al.* (49).

RNA Blot Analysis. Total nucleic acids or polyadenylylated RNAs were denatured by glyoxal and dimethylsulfoxide (50), electrophoresed on 1% agarose gels, and transferred to aminothiophenylether paper (51) or to Genatran-45 nylon blotting membrane (Plasco, Woburn, MA). After transfer to nylon membranes, the filters were baked at 80°C, treated with hot (95°C) 20 mM Tris, pH 8.0, and shaken at room temperature for 10 min and then washed at 60°C for 1 hr in 0.1× standard saline phosphate/EDTA (SSPE; 1× SSPE = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA)/0.1% $NaDodSO_4$. This wash step before prehybridization increased the resulting hybridization signal 4- to 5-fold. The filters were placed in prehybridization buffer containing 5× SSPE (52)/50% formamide (vol/vol)/5× Denhardt's reagent (0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin; ref. 53)/1% $NaDodSO_4$ containing 200 μg of denatured herring sperm DNA per ml and incubated at 42°C for 16 hr. The hybridization solution was the same as the prehybridization buffer except DNA probes were included. Probes were prepared from DNA restriction fragments by the random hexamer-primed synthesis method of Feinberg and Vogelstein (54) to a specific activity of 0.5–1.0 × 10^9 cpm/μg. The radiolabeled DNA probes were present in the hybridization solution at a concentration of 1–2 × 10^6 cpm/ml. Hybridizations were done for 36–48 hr at 42°C. The filters were washed at 42°C in 5× SSPE/50% formamide/0.2% $NaDodSO_4$ for 1 hr and then in 1× SSPE/0.1% $NaDodSO_4$ at 50°C until a low background signal was achieved. Alternately, for high-stringency washing conditions, the wash protocol was 0.1× SSPE/0.1% $NaDodSO_4$ at 60°C for 1 hr. The filters were wrapped in SaranWrap and exposed to Kodak XAR-5 film with a DuPont Cronex Lightning Plus intensifying screen at −70°C. For removal of the probe, hot (95°C) buffer containing 0.1× SSPE/0.1% $NaDodSO_4$ was poured onto the filter, and the filter was shaken slowly for 10 min at room temperature. This procedure was then repeated twice more or was further continued until an adequate amount of the probe was removed.

Preparation of Carrot DNA and Construction of Genomic Libraries. Carrot genomic DNA was prepared from the cell line WOO1C essentially as described for corn seedling DNA (55). Briefly, carrot cells were disrupted in a Dounce homogenizer (A pestle) in ice-cold buffer (15% sucrose/50 mM Tris·HCl, pH 8/50 mM Na_3EDTA/0.25 M NaCl). The homogenate was centrifuged at 2000 × *g* for 10 min at 4°C. The pellet was resuspended in 15% sucrose/50 mM Tris·HCl, pH 8/50 mM Na_3EDTA at 0°C and *N*-lauroylsarcosine was added to a concentration of 1% (wt/vol). The solution was mixed gently, and CsCl was added. Insoluble material was removed by centrifugation at 22,000 × *g* for 20 min at 4°C. Ethidium bromide was added, and the DNA was purified by two cycles of equilibrium density centrifugation (53). The carrot PAL and HRGP genes were isolated from a genomic EMBL-3 library (56) constructed from size-fractionated *Sau*3A-partially digested restriction fragments. For isolation of the carrot PAL-encoding gene, a library containing 0.8 × 10^6 recombinants was screened using a radiolabeled parsley PAL cDNA clone (13). For isolation of additional carrot HRGP clones, a library containing 1 × 10^6 recombinants was screened using a carrot HRGP gene (57). As a control for RNA loading in RNA blotting analysis, a carrot tubulin cDNA (gift of R. Sung, University of California, Berkeley) was used as a hybridization probe.

RESULTS

To examine the role of ethylene in the activation of plant defense responses, the effect of ethylene on the expression of four plant defense-response genes was examined in carrot (*Daucus carota*). PAL, 4-CL, and CHS are key branch-point enzymes in the biochemical pathway of phytoalexin and lignin synthesis, whereas HRGPs may perform a structural role in plant defense responses. A cDNA (13) encoding a portion of the parsley PAL mRNA was used as probe to isolate the homologous PAL gene from carrot (58). The parsley and carrot PAL genes are quite homologous [as determined by Southern blot hybridization (58)], a degree of homology not unexpected because carrot and parsley are members of the same family, *Umbelliferae*. The carrot PAL gene was used as probe in RNA blot hybridization experiments designed to examine the effect of ethylene on PAL mRNA accumulation. As shown in Fig. 1*A*, 10 ppm ethylene induced the accumulation of PAL mRNA in carrot roots. Similarly, cDNAs (13) encoding portions of the parsley 4-CL and CHS mRNAs were used as hybridization probes in RNA blot experiments. Ethylene increases the steady state levels of 4-CL mRNA (Fig. 1*B*) and CHS mRNA (Fig. 1*C*), 20- and 50-fold, respectively. The time of maximal accumulation of each of these mRNAs differed; 4-CL was maximally induced

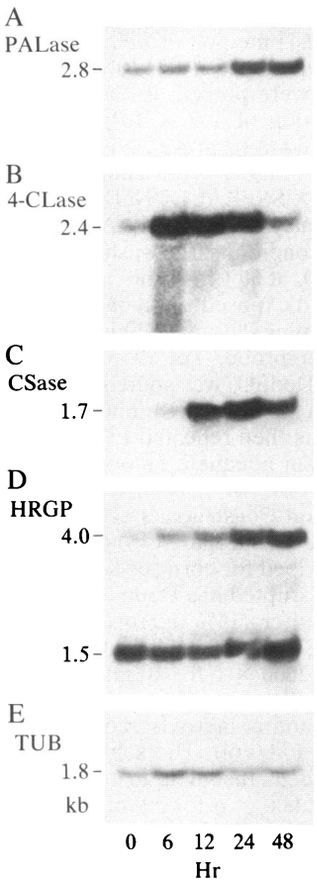

FIG. 1. Time course of mRNA accumulation for several plant defense genes in response to ethylene. Whole actively growing carrot roots were placed in 4-liter jars and allowed to equilibrate in a stream of moist air. After 24 hr the roots were flushed at a rate of 100 ml/min with 10 ppm ethylene in hydrocarbon-free air. At various times during the treatment groups of four to six carrots were removed, and polyadenylylated RNA was prepared. Polyadenylylated RNA was denatured by glyoxal, separated on 1% agarose gels, and transferred to aminothiophenylether paper as previously described (49). The same blot was probed under stringent conditions (hybridization: 50% formamide/5X SSPE, 42°C; wash: 0.1X SSPE/0.1% $NaDodSO_4$) with either the carrot PAL gene (*A*), a parsley 4-CL cDNA (*B*), a parsley CHS cDNA (*C*), a carrot HRGP gene (*D*), or a carrot α-tubulin (TUB) cDNA (*E*). After each use, the same blot was stripped and rehybridized with a different probe.

by 6 hr, CHS by 24 hr, whereas PAL mRNA continued to increase even after 48 hr.

A carrot HRGP gene (57) was used as a hybridization probe in similar RNA blot experiments. A complex pattern of regulation of HRGP mRNAs was evident. Three species of mRNAs of sizes 1.5, 1.8, and 4.0 kilobases (kb) were present in untreated carrot roots (Fig. 1*D*). There was a moderate level of the 1.5-kb HRGP transcript and a low level of the 1.8- and 4.0-kb transcripts in rapidly growing roots. After 48-hr exposure to 10 ppm ethylene, a 50- to 100-fold increase in the level of the 4.0-kb mRNA occurred. The 1.5- and 1.8-kb HRGP mRNAs were only slightly affected by ethylene in rapidly growing roots; their regulation in stored roots is discussed below. The steady state level of a control mRNA (α-tubulin) was unaffected by ethylene (Fig. 1*E*). Additional experiments reveal that after as little as 1 hr of ethylene treatment, an increase in the steady state levels of PAL, 4-CL, and HRGP mRNAs was observed in whole carrot roots. A carrot root is a complex organ (59); the type and number of cells in the root responding to ethylene are unknown. If only a low percentage of cells in the entire root are responding to ethylene, then at the cellular level, these ethylene-induced increases in plant defense gene mRNAs could be much larger than indicated by an analysis of whole root mRNAs.

Pathogenic infection of plants has been shown to increase the rate of respiration and its associated enzymes (60). Moreover, Kombrink and Hahlbrock (14) recently reported that elicitor treatment of parsley cells increases the level of enzymes involved in respiration. Interestingly, treatment of plants with ethylene also causes an increase in the respiration rate (61). The ethylene-induced increase in the rate of respiration is further potentiated by increased oxygen levels (61). To determine whether the ethylene-induced levels of PAL, 4-CL, CHS, or HRGP mRNAs could be further potentiated by oxygen, the effect of 10 ppm ethylene in the presence of 100% oxygen on PAL, 4-CL, and HRGP mRNAs was examined. Analysis of RNA prepared from carrot roots treated with ethylene in oxygen revealed that mRNAs encoding the 4-CL (Fig. 2*B*) and CHS (data not shown) enzymes accumulate to a higher steady state level and remain at a higher level in the presence of oxygen. However, accumulation of PAL (Fig. 2*A*), HRGP (Fig. 2*C*), or α-tubulin (Fig. 2*D*) mRNAs was not further increased by an oxygen-enriched atmosphere. Oxygen alone has no effect on any of the above-mentioned mRNAs (data not shown).

As previously discussed, exposure to 10 ppm ethylene causes a dramatic 50- to 100-fold increase in the level of the 4.0-kb HRGP mRNA in both rapidly growing (Fig. 1*D*) and stored carrot roots (Fig. 2*C*). However, unlike the case for rapidly growing roots, a 1.8-kb HRGP mRNA was induced after ethylene treatment of stored roots, whereas a 1.5-kb HRGP transcript decreased in abundance during the same time period (Fig. 2*C*). Further examination of Fig. 2*C* reveals that there is only a relatively small increase (2- to 4-fold) in

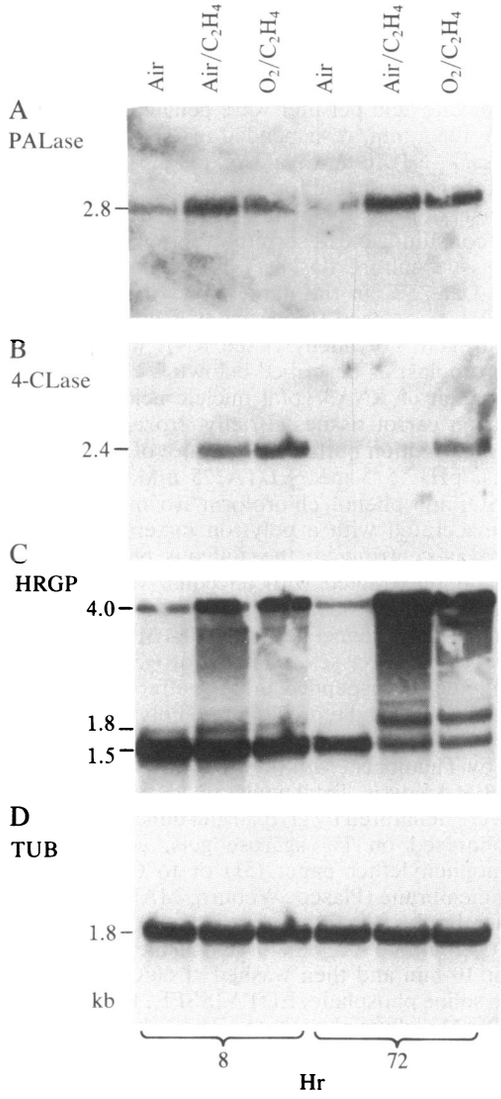

FIG. 2. Effect of ethylene in the presence of oxygen on several plant defense gene mRNAs. Polyadenylylated RNA was prepared from whole stored carrot roots treated for 8 or 72 hr with air or 10 ppm ethylene in air or 10 ppm ethylene in pure oxygen. An RNA blot of glyoxal-treated RNAs was probed as described in Fig. 1 with either the carrot PAL gene (*A*), a parsley 4-CL cDNA (*B*), a carrot HRGP gene (*C*), or a carrot α-tubulin (TUB) cDNA (*D*).

the total amount of HRGP-homologous sequences present in ethylene-treated versus untreated roots. This is due to the relatively high level of 1.5-kb mRNA compared with the 1.8-kb and 4.0-kb HRGP mRNAs in untreated roots. However, there is a dramatic change in the type of mRNA produced during ethylene treatment; there is a shift in accumulation of HRGP mRNAs from the 1.5-kb mRNA to 4.0-kb mRNA. These results could explain why only relatively small increases in proline incorporation into plant cell wall HRGPs were detected following ethylene treatment or fungal infection of melon (62, 63). An additional ethylene-induced mRNA (2.0 kb) homologous to the HRGP probe was also detected after 72 hr (Fig. 2*C*). This transcript may either have low homology to the HRGP gene probe and/or is a very low-abundance transcript. In support of the former possibility, several additional carrot λ genomic clones weakly homologous to the HRGP gene probe have been isolated.

Upon wounding of stored carrot root, there is a dramatic increase in accumulation of the 1.5-kb HRGP mRNA relative to other size HRGP mRNAs. Within 1 hr, the level of 1.5-kb mRNA increased over 10-fold, while the level of 1.8-kb mRNA decreased (Fig. 3). This wound-induced accumulation of a 1.5-kb HRGP mRNA is very large in stored roots but only several-fold in rapidly growing roots and cell cultures (ref. 30 and data not shown). The high basal level of 1.5-kb HRGP mRNA in developing plant tissues may reflect the requirement of this HRGP protein for plant growth. The effect of wounding on the 4.0-kb HRGP mRNA was not examined. Similarly, the process of protoplast preparation of carrot cells resulted in the induction of a 1.5-kb HRGP mRNA (Fig. 4). Although the exact physiological state of protoplasts is unknown, the induction of HRGP mRNA in protoplasts mimics a wounding effect.

DISCUSSION

Pathogen attack and elicitor treatment dramatically increase the rate of ethylene production in plants. Subsequently, phytoalexins, lignin, chitinase, and incorporation of hydroxyprolinerich glycoproteins into plant cell walls increase in these stressed plant tissues. For example, infection of melon (*Cucumis melo*) with the pathogen *Colletotrichium lagenarium* or treatment with elicitors from either fungal or plant origin results in increased ethylene production (64) followed by a dramatic increase in chitinase activity (22) and deposition of hydroxyproline in the cell wall (63). In the presence of an inhibitor of ethylene biosynthesis, the dramatic increases in chitinase (22) activity and the deposition of hydroxyproline (63) in the cell wall of infected tissues fail to occur. Furthermore, addition of ACC, the immediate precursor of ethylene, to melon tissues induces ethylene production and concomitantly increases chitinase activity (22) and hydroxyproline incorporation into cell walls (63). Similarly, Broglie *et al.* (23) have reported that ethylene treatment of bean seedlings results in a dramatic increase in steady-state chitinase mRNA levels. These results suggest that the induction of chitinase and hydroxyproline incorporation into wall proteins by pathogen attack may result, in part, from triggering of ethylene production. Moreover, as a gas, locally produced ethylene is eminently suited to induce defense-response genes in both neighboring and somewhat distant plant tissues in advance of pathogen movement. Lending support to this idea are several recent reports that infection of bean (*Phaseolus vulgaris*) hypocotyls with the fungal pathogen *Colletotrichum lindemuthianum* induces defense-response mRNAs not only in cells at the infection site but also at uninfected sites distantly located (16, 33). We have demonstrated a more direct link between ethylene and new enzyme production by showing that large increases in mRNA levels for the plant defense response genes, PAL, 4-CL, CHS, and HRGPs occur in response to the plant stress hormone ethylene. Together, these results strongly support the proposal that ethylene produced in response to biological stress is a signal for plants to activate defense mechanisms against potential pathogens.

The effect of wounding on HRGP mRNA levels contrasts with that of ethylene. Ethylene causes an increase in the steady state levels of 1.8- and 4.0-kb HRGP mRNAs. The steady state level of a 1.5-kb HRGP mRNA is increased by wounding, whereas ethylene causes a reduction in abundance of this transcript. These results indicate that the 1.5- and 1.8-kb HRGP transcripts are differentially regulated. Both 1.5- and 1.8-kb HRGP mRNAs have been mapped to a single

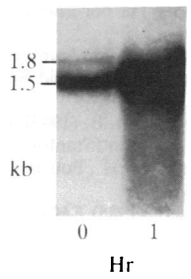

FIG. 3. Effect of wounding on the accumulation of certain HRGP mRNAs. Whole stored carrot roots were peeled such that the upper 1–2 mm was removed and the peel was discarded. The next 1–2 mm of root tissue was quickly peeled directly into liquid nitrogen (t_0), and the roots were incubated in a stream of moist air. After 1 hr, the next 1–2 mm of root tissue was peeled into liquid nitrogen (1-hr time point), and the remainder of the root was discarded. Total RNA (40 μg), prepared from the 0- and 1-hr time points, was treated with glyoxal, separated on a 1% agarose gel, blotted to a Genatran-45 nylon membrane, and probed with the carrot HRGP gene as described. A carrot tubulin cDNA probe was used as a control for equal loading of RNA (data not shown).

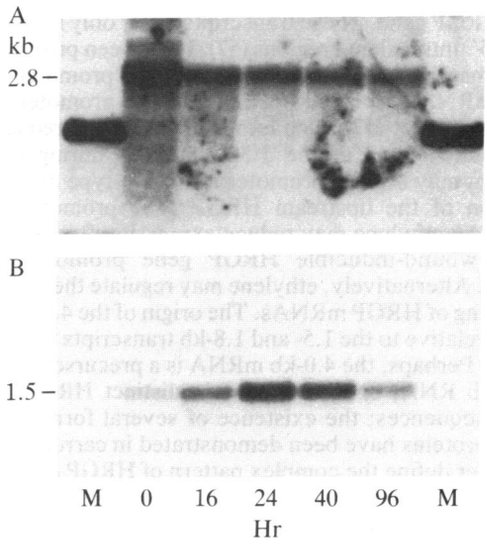

FIG. 4. Effect of protoplast formation on PAL and HRGP mRNAs. Carrot cell cultures were treated with 2% Driselase in 10 mM NaOAC, pH 5.5/50 mM $CaCl_2$/0.6 M sorbitol for 3 hr. Protoplasts were washed several times in buffer without enzyme and then cultured in MS medium containing 0.1 mg of 2,4-d dichlorophenoxyacetic acid per liter and 0.6 M sorbitol. At various times (indicated above) polyadenylylated RNA was prepared from a portion of the protoplasts (t_0 is mRNA from control cells). Glyoxal-treated mRNAs (10 μg) were separated on a 1.0% agarose gel, blotted to aminothiophenylether paper, and probed with radiolabeled PAL gene (*A*) or the gene HRGP (*B*). Lanes M (markers) show hybridization of the 1.6 kb *Hinf* I fragment of pUC18 to vector sequences in the probe.

carrot HRGP gene. These transcripts differ only in the length of their 5′ untranslated regions (57). It has been proposed that each transcript has its own promoter; the promoter for the 1.8-kb mRNA is located upstream of the promoter for the 1.5-kb transcript (57). One explanation for the reduction in steady-state levels of the 1.5-kb mRNA during ethylene treatment may be by a promoter occlusion-type mechanism. Induction of the upstream HRGP gene promoter (1.8-kb mRNA) by ethylene may reduce expression from the downstream wound-inducible HRGP gene promoter (1.5-kb mRNA). Alternatively, ethylene may regulate the differential processing of HRGP mRNAs. The origin of the 4.0-kb HRGP mRNA relative to the 1.5- and 1.8-kb transcripts is at present unclear. Perhaps, the 4.0-kb mRNA is a precursor to the 1.5- or 1.8-kb RNA, or it may contain distinct HRGP protein-coding sequences; the existence of several forms of carrot HRGP proteins have been demonstrated in carrot roots (65). To further define the complex pattern of HRGP regulation, a λgt10 cDNA library from ethylene-treated carrot root mRNA has been constructed, and cDNAs corresponding to the 4.0-kb, 1.8-kb, and 1.5-kb HRGP mRNAs have been identified. Analysis of the DNA and protein sequences encoded by these differentially expressed cDNAs may provide a clue as to their different roles in plant stress responses.

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